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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

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TITLE OF THE INVENTION (280 character maximum)

DOUBLE-STRANDED RIBONUCLEIC ACID WITH INCREASED EFFECTIVENESS IN AN ORGANISM

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METHOD OF PAYMENT

X	A check or money order is enclosed to cover the Provisional Filing Fee	PROVISIONAL FILING FEE AMOUNT ($)	$80.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

X No. ___ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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Additional inventors, if any, are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

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Application of:	Vornlocher, et al.
Serial No.:	Not Yet Assigned
Filed:	June 18, 2003
Entitled:	DOUBLE-STRANDED RIBONUCLEIC ACID WITH INCREASED EFFECTIVENESS IN AN ORGANISM

CERTIFICATE OF MAILING UNDER 37 CFR 1.10

I hereby certify that the paper (and any paper or fee referred to as being enclosed) is being deposited with the United States Postal Service using Express Mail to Addressee Service, under 37 C.F.R. Section 1.10, **Express Mail Label No. EV242752015US** on this date, **June 18, 2003**, postage prepaid, in an envelope addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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TRANSMITTAL LETTER

Enclosed for filing in the above-identified provisional patent application, please find the following documents:

1. Cover Sheet for filing Provisional Application;
2. Provisional Patent Application Specification;
3. One sheet of informal drawings;
4. Application Data Sheet
5. Check in the amount of $80.00 for the requisite filing fee; and
6. Return Post Card.

Pursuant to 37 C.F.R. §1.27, Applicant claims small entity status.

The Commissioner for Patents is hereby authorized to charge any additional fees or credit any overpayment in the total fees to Deposit Account No. 16-0085, Reference No. 20200/2141. A duplicate of this transmittal letter is enclosed for this purpose.

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Date: June 18, 2003

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Double-Stranded Ribonucleic Acid with Increased Effectiveness in an Organism

The invention concerns a double-stranded ribonucleic acid (dsRNA) consisting of two single strands, having increased effectiveness in an organism; a method for its targeted selection; a medicament containing this dsRNA; and a use of such a double-stranded ribonucleic acid.

It is known that dsRNA can be used to inhibit the expression of a target gene by means of RNA interference. To date, the dsRNA used has been of varying effectiveness. A correlation between the effectiveness of a dsRNA, its length, and the position and length of overhangs of unpaired nucleotides is known from WO 02/44321 A2. It has been determined that in a 2-nucleotide-long overhang situated at the 3'-end of a strand of dsRNA, efficiency is particularly good when the next-to-last nucleotide at the 3'-end is a U.

The task of the present invention is to make available a method for the targeted selection of a dsRNA that exhibits increased effectiveness in an organism to inhibit the expression of a target gene by means of RNA interference, a use of such a dsRNA, such a dsRNA, and a medicament containing such a dsRNA.

The task is solved by the elements in Claims 1, 9, 17, and 18. Advantageous enhancements result from the elements in Claims 2 to 8 and 10 to 16.

In terms of the invention, a method is anticipated for the targeted selection of a double-stranded ribonucleic acid (dsRNA) consisting of two single strands that exhibits increased effectiveness in inhibiting the expression of a target gene by means of RNA interference, whereby the sequences of the single strands of the dsRNA are selected in such a way that on both ends of the dsRNA the last complementary nucleotide pair is G-C, or at least two of the last four complementary nucleotide pairs are G-C pairs; whereby the dsRNA exhibits a single-stranded overhang consisting of 1 to 4 unpaired nucleotides at the first end, and no overhang at the

second end; whereby the unpaired nucleotide of the single-stranded overhang that is directly adjacent to the last complementary nucleotide pair contains a purine base, excluding the following dsRNAs:

5'- CAG GAC CUC GCC GCU GCA GAC C-3' (SEQ ID NO: 1)
3'-CG GUC CUG GAG CGG CGA CGU CUG G-5' (SEQ ID NO: 2),

5'- G CCU UUG UGG AAC UGU ACG GCC-3' (SEQ ID NO: 3)
3'-UAC GGA AAC ACC UUG ACA UGC CGG-5' (SEQ ID NO: 4),

5′- CUUCUCCGCCUCACACCGCUGCAA *3′ (SEQ ID NO: 5)
3′- GAAGAGGCGGAGUGUGGCGACG (SEQ ID NO: 6)

"G," "C," "A" and "U" each stand for a nucleotide that contains guanine, cytosine, adenine, and uracil as a base, respectively. In general, the "target gene" is taken to mean a DNA strand of the double-stranded DNA in the cell, which is complementary to a segment of the other DNA strand of the double-stranded DNA, and which serves as a matrix during transcription. The segment contains all transcribed regions. In the target gene this is generally the sense strand. Thus an antisense strand of the dsRNA can be complementary to an RNA transcript formed during expression of the target gene, or to its processing product, such as an mRNA. A sense strand of the dsRNA is the strand of the dsRNA that is complementary to the antisense strand. A dsRNA is present when the ribonucleic acid consisting of two ribonucleic acid strands exhibits a double-stranded structure. Not all nucleotides of the dsRNA must exhibit Watson-Crick base pairings. In particular, single base pairs that are not complementary do not compromise the effectiveness of the dsRNA in inhibiting expression by means of RNA interference, or do so very little.

The sequences of the single strands of the dsRNA can be selected by selecting a region and its length within the target gene to be inhibited, such that a dsRNA with a strand that is complementary to it exhibit the above-described elements. Because single nucleotides that are not complementary to the target gene do not inhibit RNA interference, it is possible to attach a single nucleotide or single nucleotides to the region of a strand of dsRNA that is complementary to the target gene, or to replace individual nucleotides in the strand in order to obtain a dsRNA that exhibits the elements defined in the terms of the invention.

In studying effectiveness, WO 02/44321 A1 failed to consider the fact that the effectiveness of a dsRNA in medical application in an organism is also dependent on its bioavailability. This increases the more stable the dsRNA is in the blood, and thus the longer the dsRNA remains available. The stability of dsRNA in the blood is in particular determined by its degradability by enzymes present in the blood. Surprisingly, it has been shown that this degradability is dependent on the sequences of the single strands that form the dsRNA. As a result of the method that is the subject of this invention, dsRNA having greater stability in the blood, and therefore greater bioavailability than another dsRNA, can be selected. Because the measurement of stability in the blood is approximated experimentally by determining the stability in serum, that is, in the aqueous phase of the blood freed of cellular components and coagulation factors, this stability will herein be designated as serum stability. However, this formulation is in no way limiting.

Furthermore, the formulation "a double-stranded ribonucleic acid consisting of two single strands," as used herein does not exclude the possibility of a bond between the single strands. This formulation was chosen in order to clarify that partially self-associated single strands (stem loops) are excluded. Both single strands of the ribonucleic acid can, however, be bound, for example, by one or preferably several chemical bonds, whereby stability is further increased. For example, the 5'-end of the antisense strand can be bound by a hexaethylene glycol linker with the 3'-end of the sense strand. Researchers are aware of many potential ways of further stabilizing dsRNA by means of such bonds.

The chemical bond may be achieved either by a covalent or ionic bond, a hydrogen bond, hydrophobic interaction, preferably by means of van der Waals or stacking interactions, or by means of metal-ion coordination. It can be produced in accordance with a particularly advantageous enhancement at at least one, preferably at both, ends.

It has furthermore been shown to be advantageous when the chemical bond is formed by means of one or several bonding groups, whereby such bonding groups are preferably poly-(oxyphosphinicooxy-1,3-propandiol)- and/or polyethylene glycol chains. The chemical bond

can also be formed by means of purine analogs used in the double-stranded structure instead of purines. It is furthermore advantageous if the chemical bond is formed by azabenzene units introduced into the double-stranded structure. It can also be formed by branched nucleotide analogs instead of nucleotides used in the double-stranded structure.

It has been shown to be advisable that at least one of the following groups is utilized in producing the chemical bond: methylene blue; bifunctional groups, preferably bis-(2-chlorocthyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; psoralen. Further, the chemical bond can bc formed by thiophosphoryl groups introduced at the ends of the double-strandcd rcgion. The chemical bond at the ends of the double-stranded region is preferably produccd by triple-helix bonds.

The chemical bond can bc suitably induced by ultraviolet light.

The nucleotides of the dsRNA can be modified. This acts to counter the activation of protein kinase, PKR, that is dependent on the doublc-strandcd RNA. Preferably, at least one 2'-hydroxyl group of the nucleotides of thc dsRNA in the double-stranded structure is replaced by a chemical group, preferably a 2'-amino- or a 2'-methyl group. At least one nucleotide in at least one strand of the double-stranded structurc can also be a so-called "locked nucleotide" with a sugar ring, preferably chemically modificd by a 2'-O, 4'-C methylene bridge. It is an advantageous for several of the nucleotides to be "lockcd nucleotides."

The excluded dsRNAs are already known, without their serum stability, the improved effectiveness associated with it, or a correlation between the sequences of their single strands and their serum stability or effectiveness, respectively, having been determined.

The advantage of the method that is the subject of this invention consists therein that it enables a dsRNA to be made available that is relatively stable in the blood and that exhibits greater effectiveness than other dsRNAs as a result longer availability. A single-stranded overhang in the dsRNA contributes to an increase in the intracellular effectiveness of the dsRNA. The stability of this dsRNA is also increased when no overhang is present at the second end of the dsRNA.

DsRNA is rendered particularly stable in the blood when the unpaired nucleotide of the single-stranded overhang that is directly adjacent to the last complementary nucleotide pair contains a purine base. This purine base can be guanine or adenine. This contradicts the lesson of WO 02/44321 A2, that it is particularly advantageous for a 2-nucleotide-long overhang at the 3'-end of a strand of the dsRNA when the next-to-last nucleotide is at the 3'-end, and therefore that the unpaired nucleotide that is directly adjacent to the last complementary nucleotide pair is a U, i.e., a pyrimidine base.

The overhang preferably consists of only one or two unpaired nucleotides. In a single-stranded overhang consisting of more than one nucleotide, stability is further increased when at least half of the overhang consists of purine bases, in particular of nucleotides containing G or A. It is particularly advantageous if the overhang exhibits the sequences 5'-GC-3'.
Even better effectiveness combined with high serum stability is achieved when the first end of the dsRNA is situated at the 3'-end of an antisense strand of the dsRNA, and the second end of the dsRNA is situated at the 3'-end of a sense strand of the dsRNA. In this case, the dsRNA only exhibits a single-stranded overhang at the 3'-end of the end of the antisense strand, while the end of the dsRNA situated at the 3'-end of the sense strand of the dsRNA exhibits no overhang.

It has furthermore been shown to be advantageous with regard to effectiveness when a region of the antisense strand of the dsRNA that is complementary to the target gene exhibits 20 to 23, in particular 22, nucleotides. Furthermore, it is advantageous when the antisense strand exhibits fewer than 30, preferably fewer than 25, particularly preferably 21 to 24 nucleotides.

Furthermore, the object of the invention is a double-stranded ribonucleic acid (dsRNA) consisting of two single strands, having increased effectiveness in inhibiting the expression of a target gene by means of RNA interference, whereby the sequences of the single strands of the dsRNA are selected in such a way that on both ends of the dsRNA the last complementary nucleotide pair is a G-C, or at least two of the last for complementary nucleotide pairs are G-C pairs; whereby the dsRNA exhibits a single-stranded overhang consisting of 1 to 4 unpaired nucleotides at the first end, and no overhang at the second end; whereby the unpaired nucleotide of the single-stranded overhang that is directly adjacent to the last complementary nucleotide pair contains a purine base, excluding the following dsRNAs:

S2: 5'- CAG GAC CUC GCC GCU GCA GAC C-3' (SEQ ID NO: 1)
S1: 3'-CG GUC CUG GAG CGG CGA CGU CUG G-5' (SEQ ID NO: 2)

S2: 5'- G CCU UUG UGG AAC UGU ACG GCC-3' (SEQ ID NO: 3)
S1: 3'-UAC GGA AAC ACC UUG ACA UGC CGG-5' (SEQ ID NO: 4)

S2: 5′- CUUCUCCGCCUCACACCGCUGCAA-3′ (SEQ ID NO: 5)
S1: 3′- GAAGAGGCGGAGUGUGGCGACG (SEQ ID NO: 6)

Advantageous enhancements of the dsRNA result from the above-described elements. Furthermore, the invention concerns the use of a dsRNA in the terms of this invention to inhibit the expression of a target gene by means of RNA interference, in particular in vitro. Over and above that, the invention concerns a medicament to inhibit the expression of a target gene by means of RNA interference, whereby the medicament contains a double-stranded RNA that is the subject of this invention, consisting of 2 single strands, and having increased effectiveness.

The invention is illustrated by an example as follows. It shows:

Fig. 1 a gel electrophoretic separation of a dsRNA that is the subject of this invention without incubation, and after 0, 15, 30, 60, 120, and 240 minutes incubation in serum,

Fig. 2 a gel electrophoretic separation of another dsRNA that is the subject of this invention without incubation, and after 0, 15, 30, 60, 120, and 240 minutes incubation in serum, and

Fig. 3 a gel electrophoretic separation of a conventional dsRNA without incubation, and after 0, 15, 30, 60, 120, and 240 minutes incubation in serum.

RNA synthesis:

Single-stranded RNAs were produced by solid phase synthesis using an Expedite 8909 synthesizer (Applied Biosystems, Applera Deutschland GmbH, Frankfurter Str. 129b, 64293 Darmstadt, Germany). Other standard ribonucleoside phosphoramidites and nucleosides immobilized on CPG (controlled pore glass), a porous support material, were obtained from ChemGenes Corp. (Ashland Technology Center, 200 Homer Ave., Ashland, MA 01721), or from Proliga Biochemie GmbH (Georg Hyken Str. 14, Hamburg, Germany). Other synthesis reagents were obtained from the Mallinckrodt Baker Co. (Im Leuschnerpark 4, 64347 Griesheim, Germany). Raw synthesis products were purified with HPLC (System Gold, Beckman Coulter GmbH, 85702 Unterschleissheim, Germany) using an anion exchange column (DNAPac PA 100, Dionex GmbH, Am Wörtzgarten 10, 65510 Idstein). The achieved yield was determined by means of UV light absorption at 260 nm.

The RNAs used in the study were produced by heating equimolar quantities of single-stranded sense- and antisense RNAs in annealing buffer (100 mM NaCl, 20 mM Na_3PO_4, pH 6.8) to 90 ± 5°C and then cooling them slowly to room temperature over approximately 3 hours.

Extraction of human serum:

For coagulation, a blood sample was immediately incubated in a darkened collecting tube (SST Vacutainer 9.5 ml; BD Vacutainer Systems, Becton Dickinson & Co., Belliver Industrial Estate, Plymouth PL6 7BP, Great Britain) for 2 hours at 20°C. After that, serum was separated

as supernatant fluid from agglutinated blood in a centrifuge at 4°C and 3000 x g for 15 minutes (Megafuge 1.0; Heraeus Instruments, Kendro Laboratory Products, 37520 Osterode, Germany), transferred to sterile 1.5 ml reagent vessels (La Fontaine, International GmbH & Co. KG, Daimlerstr. 14, 68753 Waghäusel, Germany), and stored at -20°C.

Incubation:

60 µl serum were placed on ice in each of 1.5 ml reagent vessels. Subsequently, 12 µl of a 25 µM dsRNA solution was added to each and mixed thoroughly for 5 seconds using a Vortex Genie2 (Scientific Industries, Inc., Bohemia, NY 11716). The dsRNA concentration was 4.16 µM in a volume of 72 µl. The samples were then incubated in a heat block at 37°C for 15, 30, 60, 120, and 240 minutes, and then immediately flash frozen in liquid nitrogen. One sample was flash frozen in nitrogen without incubation at 37°C immediately after dsRNA was added to the serum. The samples were stored at -80°C.

dsRNA isolation:

With the exception of a phenol solution, all reagents used for isolation were sterile-filtered and cooled on ice before use.

The samples that were stored at -80°C were placed on ice; 450 µl of a 0.5 M NaCl solution was then added to each, and mixed thoroughly after thawing for 5 seconds.

DsRNA extraction from the sample solution was done in phase lock gel reagent vessels (Eppendorf AG, 22331 Hamburg, Germany). The phase lock gel reagent vessels were then centrifuged for 2 minutes at 16,100 x g and 4°C, and then placed on ice. Subsequently, the samples were transferred to the phase lock gel reagent vessels, to which were added 500 µl of a phenol:chloroform:isoamyl alcohol mixture (Roti-Phenol, Carl Roth GmbH & Co., Schoemperlenstr. 1-5, 76185 Karlsruhe, Germany) and 300 µl chloroform. The samples were then thoroughly mixed for 30 seconds with an IKA Vibrax VXR basic, Type VX2E (IKA Works do Brasil, Ltd, Taquora, RJ 22713-000, Brazil). Subsequent phase separation was done by means of centrifugation at 4°C and 16,100 x g for 15 minutes. The upper aqueous phase was carefully transferred to a new sterile region vessel. After that, 40 µl ice-cooled 3 M

sodium acetate solution (pH 5.2) was added to the aqueous phase. The resulting solution was thoroughly mixed for 20 seconds. After the addition of 1 µl Pellet Paint (NF Co-Precipitant, Novagen, 441 Charmony Drive, Madison WI 53719) it was mixed for 5 seconds. Thereafter, 1 ml of ice-cooled ethanol was added and shaken for 20 seconds. To precipitate the dsRNA, the solution was cooled for one hour to -80°C.

The precipitated dsRNA was pelleted by means of centrifugation at 12,000 x g for 30 minutes at 4°C; the supernatant fluid was then carefully poured off, and the pellet was washed with 500 µl of ice-cooled 70% ethanol (Mallinckrodt Baker B.V., 7400 AA Deventer, Holland). After shaking for 2 seconds, it was again centrifuged at 12,000 x g and 4°C for 10 minutes, and the supernatant fluid above the pelleted dsRNA was poured off. The remaining solution was collected at the bottom of the vessel by centrifuging for 20 seconds at 16,100 x g and 4°C, and then pipetted off. The pelleted dsRNA was dried uncovered for 5 minutes at room temperature.

The dried dsRNA was then dissolved by mixing thoroughly for 2 minutes in 30 µl gel application buffer (95% v/v formamide, 10 mM EDTA, 0.025% w/v xylencyanol, 0.025% w/v bromophenol blue.

Analysis by denaturing gel electrophoresis:

Analysis of the dsRNA was done by means of denaturing polyacrylamide gel electrophoresis in 0.8-mm-thick and 200 x 280 mm sized gels with 8 M urea and 16% v/v formamide.

Composition of a gel (50 ml):

24 g urea	(99.5% p.a.; Carl Roth GmbH & Co., Schoemperlenstr. 1-5, 76185 Karlsruhe, Germany),
18 ml acrylamide	(rotiphoresis gel 29:1 [40%]; Carl Roth GmbH & Co., Schoemperlenstr. 1-5, 76185 Karlsruhe, Germany),
5 ml 10 x TBE	(1 M tris [ultra quality; Carl Roth GmbH & Co., Schoemperlenstr. 1-5, 76185 Karlsruhe, Germany] 1 M boric

acid [99.8% p.a., %; Carl Roth GmbH & Co., Schoemperlenstr. 1-5, 76185 Karlsruhe, Germany], 25 mM EDTA [Sigma-Aldrich Chemie GmbH P.O. 1120, 89552 Steinheim, Germany] in deionized water),

8 mm formamide (Merck-Schuchardt, 85662 Hohenbrunn, Germany),

50 µl temed (N,N,N',N'-tetramethyl ethylene diamine) (Sigma-Aldrich Chemie GmbH P.O. 1120, 89552 Steinheim, Germany), and

200 µl APS ammonium persulfate (10% w/v) (Gibco BRL Life Technologies, Invitrogen GmbH, Karlsruhe Technology Park, Emmy Noether Str. 10, 76131 Karlsruhe, Germany).

After pouring the gel between two glass plates and polymerizing it for approximately 30 minutes, a first run was done in a gel run apparatus for approximately 30 minutes at 45 mA (power source: Power PAC 3000; Bio Rad Laboratories 2000 Alfred Nobel Drive, Hercules, CA 94547). 1 x TBE was used as the gel running buffer. In order to equalize the temperature of the gel a 3-mm-thick aluminum plate was affixed to one of the glass plates.

Before application onto the gel, the samples were heated for 5 minutes to 100°C, chilled on ice, and centrifuged for 20 seconds at 13,000 x g and 4°C.

10 µl of each sample was applied. In addition, a dsRNA sample that was not incubated with serum (2 µl 25 µM dsRNA in 10 µl gel application buffer) was applied.

Electrophoresis was done for 90 minutes at 45 mA. Finally, the gel was stained for 30 minutes with Stains-all (40 mg Stains-all (1-ethyl-2-[3(3- ethylnaphtho[1,2-d]thiazoline-2-ylidine)-2-methylpropenyl]naphtho-[1,2-d]thiazolium bromide); Sigma-Aldrich Chemie GmbH P.O. 1120, 89552 Steinheim, Germany) + 400 ml formamide (Merck-Schuchardt, 85662 Hohenbrunn, Germany) + 400 ml H_2O), and then de-stained in a water bath for approximately

30-60 minutes. The de-stained gels were digitized using a photodocumentation apparatus (Image Master VDS Pharmacia Biotech; Amersham Biosciences Europe GmbH, Munzinger Str. 9, 79111 Freiburg; by D & R, Israel) and then scanned in color mode (Silver Fast, UMAX Technologies, Inc., 10460 Brockwood Road, Dallas, TX 75238; Adobe Photoshop Elements, Adobe Systems, Inc., 345 Park Ave., San Jose, CA 95110-2704).

Results:

The following dsRNAs were used:

1. BCL20, whose S1 antisense strand is complementary to a sequence of the sense strand of the human BCL-2 gene (Gene Bank accession number M13994):

S2: 5'- GGC GAC UUC GCC GAG AUG UCC-3' (SEQ ID NO: 7)
S1: 3'-CG CCG CUG AAG CGG CUC UAC AGG-5' (SEQ ID NO: 8)

2. B133, whose S1 antisense strand is complementary to the sense strand of the human bcl-2 gene (Gene Bank accession no. M13994):

S2: 5'- ACC GGG CAU CUU CUC CUC CCA-3'
S1: 3'-CG UGG CCC GUA GAA GAG GAG GGU-5'

3. P3, whose S1 antisense strand is complementary to the sense strand of the human PLK1 gene (Gene Bank accession no. X75932):

S2: 5'- GAU CAC CCU CCU UAA AUA UUU-3'
S1: 3'-CG CUA GUG GGA GGA AUU UAU AAA-5'

Figures 1 to 3 each show from left to right a gel electrophoretic separation of a dsRNA without and after 0, 15, 30, 60, 120, and 240 minutes of incubation in serum. Figure 1 shows the gel electrophoretic separation of BCL20 dsRNA; Figure 2 that of B133 dsRNA; and Figure 3 that of P3 dsRNA.

Figure 1 shows that BCL20 dsRNA is hardly at all degraded during incubation.

It may be seen from Figure 2 that B133 dsRNA is degraded somewhat more quickly than is BCL20 dsRNA. The reason for this is that here the last complementary nucleotide pair at both ends of the dsRNA is not a C-G, as is ideally the case.

Conventional dsRNA, such as P3 dsRNA shown in Figure 3, is degraded almost immediately in serum. P3 dsRNA exhibits complementary G-C nucleotide pairs only at one end of the double-stranded structure.

ABSTRACT

The invention concerns a method for the targeted selection of a double-stranded ribonucleic acid (dsRNA) consisting of two single strands that exhibits increased effectiveness in inhibiting the expression of a target gene by means of RNA interference, whereby the sequences of the single strands of the dsRNA are selected in such a way that on both ends of the dsRNA the last complementary nucleotide pair is a G-C, or at least two of the last four complementary nucleotide pairs are G-C pairs; whereby the dsRNA exhibits a single-stranded overhang consisting of 1 to 4 unpaired nucleotides at the first end, and no overhang at the second end; whereby the unpaired nucleotide of the single-stranded overhang that is directly adjacent to the last complementary nucleotide pair contains a purine base.

Fig. 1

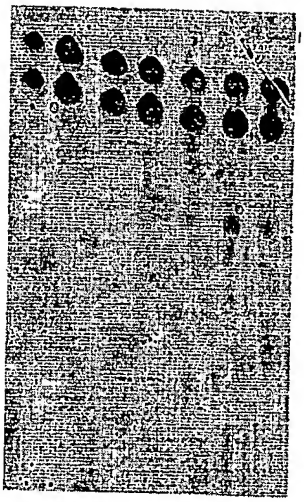


Fig. 2

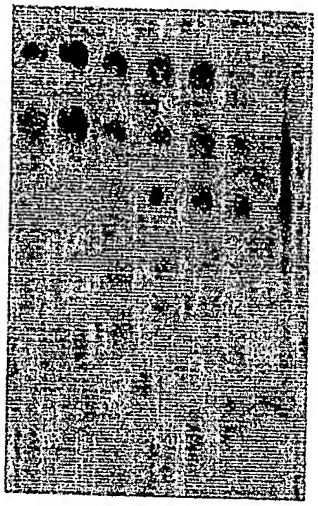
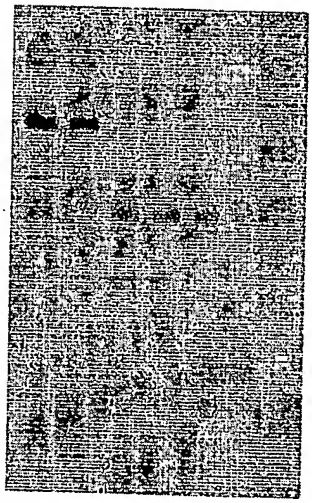


Fig. 3



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